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Our aim is to identify epitopes on the surface of prostate cells that are largely or exclusively tissue- or tumor-specific. This is done by expansion of T cells reactive for HLA-restricted, established tumor lines from the peripheral blood cells and tumor-infiltrating lymphocytes of cancer patients. Towards this end, we established a reagent bank of patient material that includes tissue, cells, and serum. We successfully and stably gene-modified the established prostate cell line, LNCaP, so that it expresses HLA-A2 and HLA-A1. We successfully transfected two other cell lines with these genes, transiently but sufficiently, for our in vitro stimulation technique. Several potentially HLA-restricted, tumor-specific T cells have been identified and are currently being cloned for retesting. In order to study tissue distribution, primary normal and tumor prostate tissue are being cultured by methods developed in our laboratory. In addition, we are performing an antibody-based method of antigen discovery that utilizes patient serum. Using this approach, several genes have been identified some of which represent previously unreported, novel genes. Studies are underway to discern the exact epitope being recognized. Studies are also underway to assess the level of gene expression in normal vs. tumor tissue from representative regions of the body.

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Michael Dalgalh 10/5/99

PI - Signature Date

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INTRODUCTION

Tumor-specific T cells, a crucial component of the immune system, have been demonstrated as able to recognize and respond to specific tumor antigens on allogeneic tumor cells in a MHC-restricted manner. Therefore, there is compelling evidence that a specific immune response in human is present against several different forms of cancer. Peripheral blood offers important benefits as a source for the generation of antigen-restricted CTL since it is easily collected from practically all cancer patients. There has been some evidence of using tumor-infiltrating lymphocytes co-cultured with irradiated tumor cells for the production of tumor-specific CTL, but there is a paucity of experimental data using this approach with peripheral blood and prostate tumor cell lines. We have leukopheresed over twenty patients with prostate cancer and have on site several established prostate tumor cell lines. These lines are being gene-modified to stably express two of the most common human leukocyte antigen (HLA) alleles occurring in North American men: HLA-A1 and –A2. Therefore, we have a source of patient material – from cells – to conduct mixed lymphocyte-tumor cell cultures for the generation of tumor-specific CTL. Also, we have a source of patient material – from sera – to use an antibody-based approach to antigen discovery that compliments our expression cloning (known as SEREX, an abbreviation for "serum expression"). Expression cloning and SEREX is being used to identify the distinct epitope on the tumor cell surface being recognized by the T-cell receptor on the CTL. Following screening of cDNA libraries by both cell- and serum-based approaches, specific genetic targets of recognition are being identified. Such loci are being sequenced to determine if a novel or previously identified epitope was detected. Using the putative protein sequence, peptides conforming to the HLA consensus binding motif for that haplotype will be synthesized. Peptides will be screening by pulsing them exogenously onto HLA-matched T2 or

autologous ebv-b cells and assessing if they stimulate antigen-specific CTL. Eventually the epitope(s) recognized by the CTL or antibodies from patient sera is identified.

BODY

Task 1. To generate $CD8^+$ T cells specifically reactive at least for the stimulating prostate cell line in a MHC-restricted fashion.

a. Gene-modify prostate tumor cell lines for stable, high expression of HLA. (see below)
b. Perform assays to determine which mixed lymphocyte tumor cell (MLTC) conditions are preferred for the generation of antigen-specific, HLA-restricted cytotoxic T lymphocytes. (see below)
c. Produce and expand sufficient amounts of effector cells for expression cloning. This series of experiments has only begun recently.

We attempted to stably transfect the gene encoding HLA-A1 and HLA-A2 into the established cell lines, LNCaP, DU145 and PC3 (Table 1, Figures 1-3). The percentage of cells expressing HLA-A2 following antibiotic selection (in G418) is shown in Table 1. In Figure 1, a representative FACS analysis shows the successful gene-modification of the prostate tumor cell line, DU145. In Figure 2, a representative FACS analysis shows the successful gene-modification of the prostate tumor cell line, PC3, as well as the maintenance of MHC class I expression following such treatment (as evidence by staining with the monoclonal antibody, BB7.2). In Figure 3, a representative FACS analysis shows the successful gene-modification of the prostate tumor cell line, LNCaP. LNCaP was easy to stably transfect; that is, high levels of HLA-A2 expression were maintain following 10 passages or greater of the original transfectant

line. DU145 and PC3 were difficult to stably transfect, as the level of expression and the precentage of cells positive for transgene expression decreased after a few passages (data not shown). If we cannot produce long-lasting HLA-A2-transfected DU145 and PC3 cells, two choices remain so as to continue our work. First, a large number of aliquots could be cryopreserved from the highly expressing initial passages. This stock could be used for in vitro stimulations for a passage or two before thawing another vial of high-expressing stock. Second, since expression remains high for several days, we could transfect the tumor lines a few days prior to their use for in vitro stimulations.

Because we have established LNCaP cells stably transfected with the HLA-A2 gene (LNCaP/A2), most of our MLTC experiments will involve this line. Should HLA-A2-modified DU145 and PC3 cells be needed, we will perform a transient transfection that should produce a high level of gene expression during MLTC coculturing as well as in ELISA.

Since setting up numerous 96-well culture conditions are both labor- and reagent-intensive, it was necessary to determine which MLTC conditions were more likely to produce HLA-restricted cloids. Therefore, for several experiments, 96-well plates were set up at various concentrations and ratios of cells. Two representative assays are shown in Table 2. Experiment one used LNCaP/A2 cells. Experiment two used PC3/A2 cells. Two 96-wells at each of the nine indicated ratios of PBMC and tumor cells were co-cultured for 10 days. Following this, microcultures were screened in singlicate for IFN-gamma secretion. Sixty microliters of each 200ul microculture was added to $5x10^4$ HLA-A2 transfected prostate tumor cells. An equal volume was added to $5x10^4$ of the parental tumor line. In general, for both experiments, plated fewer total cells resulted in specific cytokine secretion (defined as cytokine secretion against the transfected tumor line two times that of the parental line, and $\geq$100 pg/ml). The most successful

ratio was determined as 10:1; either 1) $2x10^5$ PBMC and $2x10^4$ tumor cells, or 2) $2x10^5$ PBMC and $4x10^4$ tumor cells. Optimizing MLTC conditions will permit us to set up experiments with a greater chance of generating specifically reactive cloids from the same initial number of established microwells. In the coming year, we will repeat MLTC with other prostate cell lines transiently or stably transfected with the gene for HLA-A1 or HLA-A2.

Task 2. To identify the peptide epitope responsible for immune recognition by antigen-specific, HLA-restricted T lymphocytes.

a. Produce cDNA libraries of those prostate tumor cell lines for which CTL have been generated.
b. Use ELISPOT and ELISA to screen cDNA library for that fragment which confers reactivity to antigen-specific, HLA-restricted cytotoxic T lymphocytes.
c. Screen candidate peptides from putative protein sequence using CTL admixed with exogenous pulsed readout cells to determine peptide conferring reactivity.

As part of the effort to identify the epitope recognized by T cells stimulated in vitro with DU145/A2 cells), we generated a cDNA library from DU145 poly (A+) RNA. This was initially performed in lambda phage, a portion of which was converted to plasmid (via a helper phage). We found that 88% of the library's plasmids contained cDNA inserts and the size of the inserts ranged from 0.5 to 1.6 Kb. Thus, the quality of the cDNA library was good. To determine if the DU145 cDNA library contained prostate-specific genes, we performed PCR analysis to detect PSA and PSMA, as well as two housekeeping genes GAPDH and β-actin. Indeed, we demonstrated the presence of all four genes in the DU145 cDNA library.

As a prerequisite for screening the DU145 cDNA library for cDNAs which encode antigen(s) that are recognized by DU145/A2-specific T cells, we introduced one-half of the plasmid form this library into 293 cells. The 293 cells were then selected for stable transfectants, and 25 subfractionated $G418^R$ lines isolated. We grew up these stably-transfected 293 cells and tested them for recognition by the DU145/A2-specific T cells. It was previously shown that the DU145/A2-specific T cells recognized DU145-derived epitopes in the context of both HLA-A1 and HLA-A2. Therefore, we transiently transfected 18 subfractionated lines with plasmid encoding HLA-A1, HLA-A2, or no insert. After 3 days, we co-cultivated these transfected sublines with two cloids of DU145/A2-specific T cells, collected supernatants after 48 hrs, and measured the IFN-γ released.

Using the aforementioned MLTC conditions for an initial screening, two cloids (#10 and #19) were identified as secreting IFN-gamma when co-cultured in an ELISA with DU145/A2 cells but not the DU145 parental line. We performed three independent screening assays (Table 3a-c) on the 24 DU-series sublines (293 cells stably-transfected with DU145 cDNA). After transfecting with plasmid encoding HLA-A2, -A1, or vector alone, we co-cultivated these cells with an equal number of DU145/A2-specific effector cells (cloids 10 and 19) and assayed for IFNγ secretion by ELISA. Unfortunately, these assays were difficult to interpret these assays because of the aberrant specificity of cloids 10 and 19 have exhibited when co-cultivated with DU145 cells transfecting with plasmid encoding HLA-A2, -A1, or vector alone. For cloid 10, co-cultivation with DU145/A2 cells (positive control) resulted in IFN-gamma production in only one of the three assays (Table 3a). Cloid 19 effectors have consistently released IFN-gamma when co-cultivated with untransfected DU145 cells, as well as DU145/A1 or -A2 transfectants. Work is continuing to determine whether this reactivity is specific and repeatable.

We are also generating other cloids stimulated in vitro with DU145/A2 and other transfected cell lines.

Using the methodology outlined in Figure 4 (see description below), we are also successfully building a patient-derived reagent bank, consisting of tissue (Table 4a) and tumor infiltrating lymphocytes (TILs) from prostate cancer patients (Table 4b). Most of the TIL were derived from biopsies, although a few were harvested from prostatectomy tissue. TIL were separated from tissue fragments and other contaminants via density centrifugation separation, following by cultivation in high-dose interleukin-2 (3000IU/ml). Four separate TIL lines have been generated during the first year of the grant; quite a promising result considering the paucity of reports in the literature concerning the generation of prostate-derived TIL.

TIL will supplement use of PBMC for the generation of antigen-specific T cells. In one current set of experiments, a TIL line was generated as described in Figure 4. Cells were cloned in 96-well plates and stimulated nonspecifically with anti-CD3. After two weeks in culture, 2000 wells were screened visually for growth. A total of 144 wells were positive for growth and analyzed for lytic activity specific for the prostate tumor line LNCaP/A2 by an ^{111}Indium release assay. (Donor PNCF 179 and LNCaP/A2 share expression of the class I MHC molecule A2). To test lytic activity, wells were split and half the cells were incubated with ^{111}Indium-loaded LNCaP/A2. Wells were scored as positive it the lytic activity exceeded 20%. Representative negative (clones 1-4) and all four positive wells (clones 5-8) are shown in Table 5. The four clones initially positive for lytic activity (clones 5-8) will be non-specifically expanded and retested.

We have accumulated a bank of patient serum during the past year, derived from the whole blood that often accompanies tissue samples. As a result, we decided to supplement the T

cell-based method of antigen discovery using a serological identification of antigens by recombinant expression cloning (SEREX) technology. This approach utilizes autologous patient sera to search for tumor antigens expressed in bacteria *Escherichia coli*, which are infected with lambda phages containing cDNA libraries prepared from fresh tumor tissues. Thus, this new approach bypasses both requirements for the pre-establishments of stable CTL clones or tumor-infiltrating lymphocytes (TIL), and established tumor cell lines. The use of SEREX technology in identification of human tumor antigens has been successful in many tumor types, including melanoma (1,2), renal cancer (1,3), astrocytoma (1), Hodgkin's disease (1,4), esophageal cancer (1), lung cancer (5,6), and colon cancer(7), and a number of tumor specific or associated antigens have been identified from these cancers. The tumor antigens identified by SEREX are immunogenic and are good candidates for cancer vaccines when the cognate T cell epitopes are discerned. The ability of the SEREX-identified antigen to be recognized by CTL has been demonstrated in a recent study showing that the peptide epitopes derived from a SEREX-identified tumor antigen NY-ESO-1 were recognized by CTL from a patient with high NY-ESO-1 antibody titers (8).

The overall SEREX approach is shown in Figure 5. Briefly, total cellular RNA was isolated from the cultured cells using a RNA isolation reagent. Complementary DNA expression libraries were constructed from two prostate cancer cell lines, LNCaP and PC3. Poly A+ mRNA was purified from the total cellular RNA isolated from these cells. The cDNA libraries were screened with a pool of sera from 10 patients with prostate cancer diagnosed at clinical stage D2. Membranes containing the recombinant phages were incubated with pooled patient sera, then incubated with an alkaline phosphatase (AP)-conjugated, Fc fragment-specific goat anti-human IgG. The positive plaques were visualized by staining the membranes with 4-nitro blue

tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. Following 2-3 more rounds of subcloning, positive plaques were cloned to homogeneity.

The inital screening resulted in a detection of a total of 6 positive clones from LNCaP library and 11 positive clones from PC3 library. Subsequent analyses by restriction digestion, sequencing, and individual serum reaction revealed that the 6 LNCaP-derived positive clones contained the inserts derived from 2 different genes, and the 11 PC3-derived positive clones contained the inserts representing 6 different genes. The results are summarized in Table 1. Clones were subjected to restricted digest analysis. Some clones were derived from the same gene, as indicated by the same restriction digestion patterns. Other clones showed different restriction patterns, but were found by sequencing analysis to contain the inserts representing the same gene. The different restriction patterns were due to the difference in their insert length.

The results in Table 7 show the reactivity of 10 individual patient sera from serum pool #1. For most clones, there was one patient serum that contains the antibody to the corresponding positive clone. For other clones, two patient sera were found to contain the antibody reactive to these clones. These results were in consistence with the restriction analysis of the positive clones. The same patient serum reacted to the clones derived from the same genes. Interestingly, the serum from one patient (patient #7) contained the antibodies to 3 distinct cDNA clones. No positive reaction was observed when a pool of 10 sera from normal individuals was used to react with each positive clones, and when a negative clone was used to react with each individual patient serum. This confirmed the specificity of the positive reactions observed between the positive clones and the patient sera.

All positive clones were subcloned to homogeneity, and the nucleotide sequences of the cDNA inserts were determined. The similarity of the identified clones to the known genes in the

GenBank database was searched using the BLAST similarity search program. The result is shown in Table 8. Of the 8 genes whose protein products were recognized by prostate cancer patient serum, 5 represented known genes in the GenBank database. Three clones (4, 5 and 7), were previously uncharacterized genes. Clone P1 showed a high homology to a mouse ADP-related gene, but no sequence of human homologue was found in the database.

Characterization of the three previously unknown genes is currently underway.

Task 3. Determine the range of tissue expression for the protein from which the peptide of interest is derived.

a. Use Northern blotting to assess protein expression in normal prostate tissue as well as other prostate carcinomas.

b. Use Northern blotting to assess protein expression in other tumor types.

Due to the size of the prostate, it is difficult to obtain large amounts of normal or tumor tissue for analysis. To address this problem, we are developing methods for the processing, cultivation, and characterization of the prostate tissue obtained from our affiliate, Northwest Hospital, as well as other sources (Figure 4). Although prostate tissue samples – whether normal or neoplastic – are inherently small, we are utilizing all the patient-derived materials possible. It is difficult to obtain matched normal and tumor samples from the same donor, but we have such tissue from a few patients (Table 4a). More samples are anticipated through a productive collaboration with the surgeons and pathologists of Northwest Hospital, and other collaborative institutions.

Once the tissue is processed and put into culture, we are performing characterization of these primary prostate cultures to confirm that they are prostatic in nature, and whether or not they are composed of normal, neoplastic, or mixed cells. First, we establish the composition of primary prostate cultures (epithelial vs. stromal cells). This is accomplished via an initial characterization that includes cell morphology and haplotyping. Once we have generated sufficient cells (>$2x10^6$), immunocytochemistry and flow cytometry will be performed for prostate-specific or –associated cell surface markers such as:

- Cytokeratin 8, 18 vs 5,15 (luminal vs. basal epithelial cells)
- PSA (will likely be downregulated within 2-weeks of cultivation)
- PSMA (unknown if will be downregulated)
- Androgen Receptor (not always retained by established lines)
- Prostatic acid phosphatase (PAP) (this marker is somewhat non-specific)

Follwing this, we intend to include additional evaluation that will include one or more of the following: (1) assay for the expression of prostate-specific genes by RT-PCR, (2) flow cytometric analysis (DNA per cell is quantitative and reproducible; transformed cells are often aneuploid and heteroploid), (3) karyotyping – analysis of chromosomal content is a well-defined criteria for identifying cell lines (chromosome analysis can also distinguish between normal and malignant cells, as the chromosome number is more stable in normal cells), (4) loss of specific chromosomal regions (Loss of Heterozygosity; e.g., the short arm of chr 8, the long arm of chr 16 and chr 10 frequently deleted in late-stage prostate cancer), or (5) assay for transformation (growth in soft agar).

Since the SEREX approach to antigen discovery is somewhat more progressed at this juncture, we have begun characterization and tissue expression/distribution studies of the genes

that appear to be novel. The method of analysis we are using is summarized in Figure 6. Materials are being assembled for this work. Because we are primarily interested in the potential immunogenicity of the expressed gene products, efforts have commenced to produce purified protein using the E. coli expression system. Although this method will not yield protein that undergoes post-translational modification, it is a relatively simple and straightforward means of generating protein for immunogenic analysis. In Figure 7, our method of protein production is outlined. It involves ligating inserts from the genes of interest into appropriate expression vectors (pQE-31 and pQE32). The ones we chose allow for initial screening using a chromagen to identify colonies that were successfully manipulated. An example of the initial step of this process is shown in Figure 8. The genes and expression vectors of interest have been cut with appropriate restriction enzymes, followed by standard gel electrophoretic analysis. We are currently at the stage of recovering the fragments from the gel and ligating the vector and insert. Eventually, the expressed protein will be used to stimulate T cells in vitro, in an effort to generate antigen-specific effectors.

KEY RESEARCH ACCOMPLISHMENTS

- Stable gene modification of an established prostate cell line, LNCaP, with HLA-A1 and –A2.
- Transient gene modification of the established prostate cell lines, DU145 and PC3 – with HLA-A2 – sufficient for MLTC and tumor line distribution analysis of specific T cells.
- Optimization of MLTC
- Generation of cDNA libraries from LNCaP, DU145, and PC3.
- Establishment of a human reagent bank, consisting of patient-derived tissue, cells, and sera.

- Use of T cell expression cloning to identify two potential cloids that may be specifically reactive to a certain region of LNCaP cDNA.
- Establishment of the SEREX technology as a useful means of antigen discovery. This technology did not previously exist at our institution and had to be started from scratch. Within a year, it proved successful.
- Use of SEREX to identify three novel genes whose characterization and potential immunogenicity are currently being assessed.

REPORTABLE OUTCOMES

1. This award supports the post-doctoral work of Dr. Gopi Shankar.
2. Funds from this award helped establish a human reagent bank, consisting of patient-derived tissue, cells, and sera.

CONCLUSIONS

It is possible to gene-modify tumor cells to stably or transiently express HLA. This is important when using tumor lines in any immunological studies, since the down-regulation or loss of HLA genes in such cells is a frequent occurrence. While gene-modification of tumor cells in and of itself is well established, we are extending its utility to the discovery of novel antigens. We have worked out the conditions for MLTC that should provide a greater number of potentially specific T cell cloids/clones from a given experimental set-up. This is not trivial, given the labor- and reagent-intensive nature of MLTC. Our initial efforts with T cell expression

cloning produced only two reactive cloids that, upon subsequent screening, may prove to be false positives. However, we have demonstrated the proof of principle for this approach. Due to our efforts to grow prostate-derived TIL, we have a reagent that may be more reactive to expression cloning since such lymphocytes would have had previous exposure to tumor-specific and/or associated antigens.

We anticipated the difficulties experienced in obtaining prostate-derived materials for these studies. Generating autologous tumor lines from patients whose blood samples (and, therefore, immune cells) we collected is a daunting task. As a result, when using the T cell approach to antigen discovery, one is almost exclusively limited to an allogeneic technique using established cell lines. In order to address this disadvantage, our current and future work was amended to include an antibody-based means of antigen discovery: the SEREX approach. An amount of tumor/tissue material insufficient for T cell expression cloning is often adequate for SEREX analysis. Using SEREX, we identified three novel genes not currently in gene databases. Characterization of these genes is underway – as is the production of recombinant protein for immunogenicity investigation.

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Appendix A. Tables

Table 1. Summary of the Tests of Panned HLA-A1/A2 Stably Transfected DU145, PC3, and LNCaP by Immunostaining and FACScan Analysis

Cell lines	Transfectants (A1/A2)	FACScan Results
DU145	A1	-
	A2	75%
PC3	A1	72%
	A2	-
LNCaP	A1	-
	A2	91%

Table 2.

PBMC/well	tumor cells/well	PBMC:tumor cells	no. cloids selected	
			exp. 1	exp. 2
$2x10^5$	$1x10^4$	20:1	5	1
$2x10^5$	$2x10^4$	10:1	10	12
$2x10^5$	$4x10^4$	5:1	0	3
$4x10^5$	$2x10^4$	20:1	0	0
$4x10^5$	$4x10^4$	10:1	0	8
$4x10^5$	$8x10^4$	5:1	2	1
$8x10^5$	$4x10^4$	20:1	0	2
$8x10^5$	$8x10^4$	10:1	3	0
$8x10^5$	$1.6x10^5$	5:1	0	5

Table 3a. Co-cultivation of DU145/A2 effectors with DU-series (293 cells w/ DU145 cDNA library). First ELISA.

		IFN-γ	
Cell Line	DNA	(cloid 10)	(cloid 19)
DU-4	A2	0	53
	A1	0	11
	vector	0	0
DU-7	A2	42	41
	A1	0	57
	vector	0	0
DU-8	A2	0	103
	A1	0	0
	vector	0	0
DU-10	A2	65	240
	A1	0	0
	vector	0	0
DU-12	A2	26	110
	A1	0	0
	vector	0	0
DU-13	A2	0	59
	A1	0	72
	vector	0	0
DU-15	A2	0	0
	A1	0	0
	vector	0	0
DU-16	A2	0	0
	A1	0	52
	vector	0	0
DU-17	A2	0	0
	A1	17	0
	vector	0	0
DU-23	A2	0	24
	A1	0	0
	vector	0	0
DU-24	A2	0	0
	A1	0	65
	vector	0	0
DU145	A2	20	359
	A1	0	452
	vector	0	848
293	A2	0	65
	A1	0	0
	vector	0	0
OKT3		280	above range

Table 3b. Co-cultivation of DU145/A2 effectors with DU-series (293 cells w/ DU145 cDNA library). Second ELISA.

		IFN-γ	
Cell Line	DNA	(cloid 10)	(cloid 19)
DU-4	A2	22	0
	A1	0	0
	vector	0	0
DU-7	A2	0	0
	A1	0	81
	vector	0	0
DU-8	A2	0	33
	A1	0	60
	vector	0	0
DU-10	A2	0	0
	A1	0	0
	vector	22	0
DU-12	A2	0	0
	A1	0	0
	vector	0	0
DU-13	A2	0	0
	A1	0	0
	vector	0	0
DU-15	A2	0	0
	A1	0	0
	vector	0	0
DU-16	A2	12	0
	A1	0	0
	vector	0	0
DU-17	A2	0	0
	A1	0	0
	vector	0	0
DU-23	A2	0	0
	A1	0	0
	vector	0	0
DU-24	A2	0	0
	A1	0	0
	vector	0	0
DU145	A2	0	6
	A1	0	48
	vector	0	22
293	A2	0	0
	A1	0	0
	vector	0	0
OKT3		280	above range

Table 3c. Co-cultivation of DU145/A2 effectors with DU-series (293 cells w/ DU145 cDNA library). Reactivity versus HLA-A1 not repeated. Third ELISA.

		IFN-γ	
Cell Line	DNA	(cloid 10)	(cloid 19)
DU-1	A2	0	0
	vector	0	80
DU-2	A2	0	ND*
	vector	0	ND*
DU-5	A2	0	ND*
	vector	50	ND*
DU-7	A2	188	0
	vector	159	0
DU-8	A2	ND*	0
	vector	ND*	0
DU-9	A2	0	ND*
	vector	0	ND*
DU-10	A2	0	ND*
	vector	66	ND*
DU-12	A2	0	0
	vector	0	0
DU-13	A2	ND*	0
	vector	ND*	0
DU-15	A2	0	ND*
	vector	0	ND*
DU-16	A2	ND*	0
	Vector	ND*	0
DU-21	A2	131	ND*
	vector	187	ND*
DU-23	A2	ND*	0
	vector	ND*	0
DU-24	A2	ND*	0
	vector	ND*	0
DU145	A2	55	
	A1	0	
	vector	0	
293	A2	0	
	A1	0	
	vector	0	
OKT3		387	
Medium		0	

*Not Determined

Table 3c. (cont.)

(Cloid 19)		
DU-1	A2	0
	A1	80
	vector	-15
DU-7	A2	0
	A1	0
	vector	0
DU-8	A2	0
	A1	0
	vector	0
DU-13	A2	0
	A1	0
	vector	0
DU-16	A2	0
	A1	0
	vector	0
DU-23	A2	0
	A1	0
	vector	0
DU-24	A2	0
	A1	0
	vector	0
DU145	A2	55
	A1	-13
	vector	149
293	A2	0
	A1	0
	vector	0
OKT3		387
Medium		0

Table 4a. Patient-derived epithelial cell lines being cultured.

Patient No.	Type of Tissue	Growth	Comments
1	prostate	1xT25	
2	prostate	2xT25	
3	prostate	4xT25	fibroblasts potentially taking over
4	normal prostate	3xT25 (explants) 2xT25 (growing cells)	epithelial-like cells growing well
	tumor	3xT25 (explants) 6xT25 (growing cells)	epithelial-like cells growing well
5	normal prostate tumor	2xT25 (explants) 2xT25 (explants)	
6	prostate	2xT25 (explants) 2xT25 (expanded cells)	
7	prostate	3xT25 (explants) 2xT25 (expanded cells)	
8	prostate	2xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells
9	prostate	4xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells
10	prostate	3xT25 (explants) 4xT75 (expanded cells)	epithelial/stromal mix epithelial cells
11	prostate	4xT25 (explants)	epithelial/stromal mix
12	prostate	4xT25 (explants) 2xT75 (expanded cells)	epithelial/stromal mix epithelial cells
13	prostate	3xT25 (explants)	epithelial/stromal mix

Table 4b. Patient-derived TIL in culture.

Patient No.	Type of Tissue	Aliquots Frozen (>1x10^6/vial)
1	prostatectomy	none
2	prostatectomy	none
3	prostate	2
4	prostate	1
5	prostate	5
6	prostate	none
7	prostate	2
8	prostate	none as yet (slow growth)
9	prostate	none as yet (slow growth)
10	prostate	none as yet (slow growth)
11	prostate	none as yet (slow growth)
12	prostate	none as yet (still at explant stage)
13	prostate	none as yet (still at explant stage)
14	prostate	none as yet (still at explant stage)

Table 5: Selection of Tumor Lytic TIL Clones

	CPM
Clone 1	605
Clone 2	1101
Clone 3	482
Clone 4	1388
Clone 5	**2215**
Clone 6	**1972**
Clone 7	**2425**
Clone 8	**1955**
Spontaneous Release	752
Maximal Release	4659
>20% Specific Lysis	>1533

Table 6. Numbers of the positive clones and numbers of different genes represented by the positive clones.

Source	Serum pool #	No. of serum in the pool	No. of clones screened	No. of positive clones	No. of different genes
PC3	#1	10	2×10^5	11	6
LNCaP	#1	10	2×10^5	2	1
LNCaP	#2	20	1×10^5	4	1

Table 7. Reaction of positive clones to individual patient serum

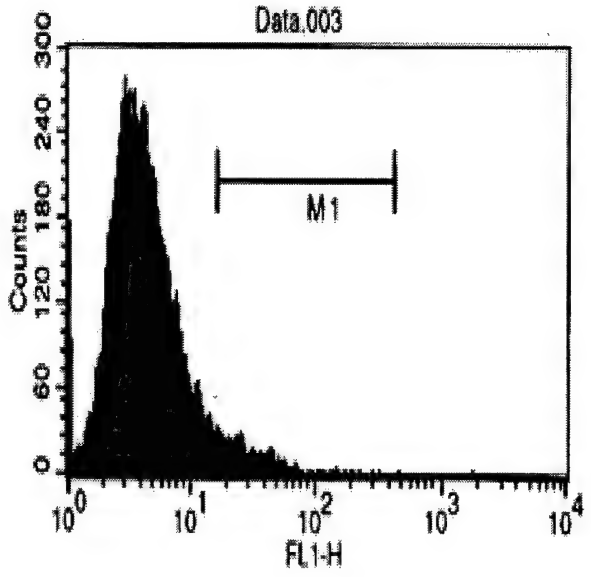
Clone #	Prostate Cancer Patient Serum (Stage D2)										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	Normal
P1							+				
P2						+					
P3						+					
P4						+					
P5						+					
P6					+						
P7		+							+		
P8						+					
P9		+							+		
P10						+					
P11		+							+		
P(-)											

Table 8. Identities of the SEREX-identified clones from prostate cancer cell lines.

Clone ID	Source	Homologous Genes in GenBank	Comments
1	PC3	Mouse ADP-related gene	No human homologue
2	PC3	Human dehydrogenase	
3	PC3	Human gene involved in translation	
4	PC3	None	New gene/ Unknown function
5	PC3	None	New gene/ Unknown function
6	PC3	Anemia-related gene	
7	LNCaP	None	New gene/ Unknown function
8	LNCaP	Human nuclear protein	

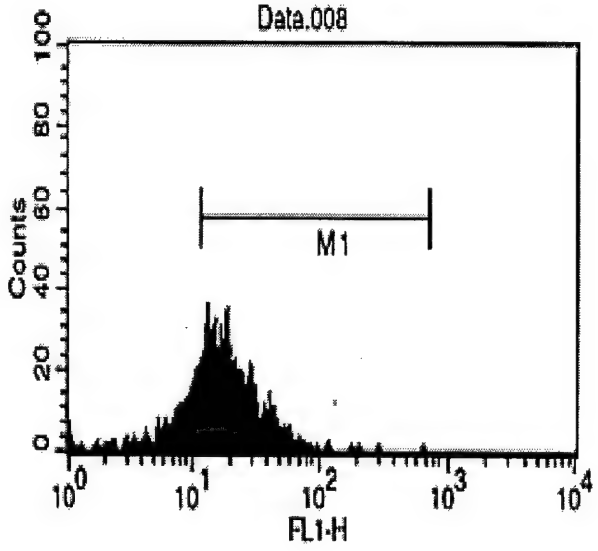
Appendix B. Figures

Figure 1. FACS Analysis of panned, transfected DU145



File: Data.003
Sample ID: DU145 parental anti HLA-A2
Acquisition Date: 15-Apr-99
Gated Events: 10000
Total Events: 10000

Marker	Events	% Total	Mean	Geo Mean
All	10000	100.00	6.76	4.49
M1	644	6.44	34.80	28.98



File: Data.008
Sample ID: DU145 panned anti HLA-A2
Acquisition Date: 15-Apr-99
Gated Events: 1000
Total Events: 1000

Marker	Events	% Total	Mean	Geo Mean
All	1000	100.00	20.68	15.82
M1	754	75.40	25.05	21.10

Figure 2. FACS Analysis of PC3 (p36) cells

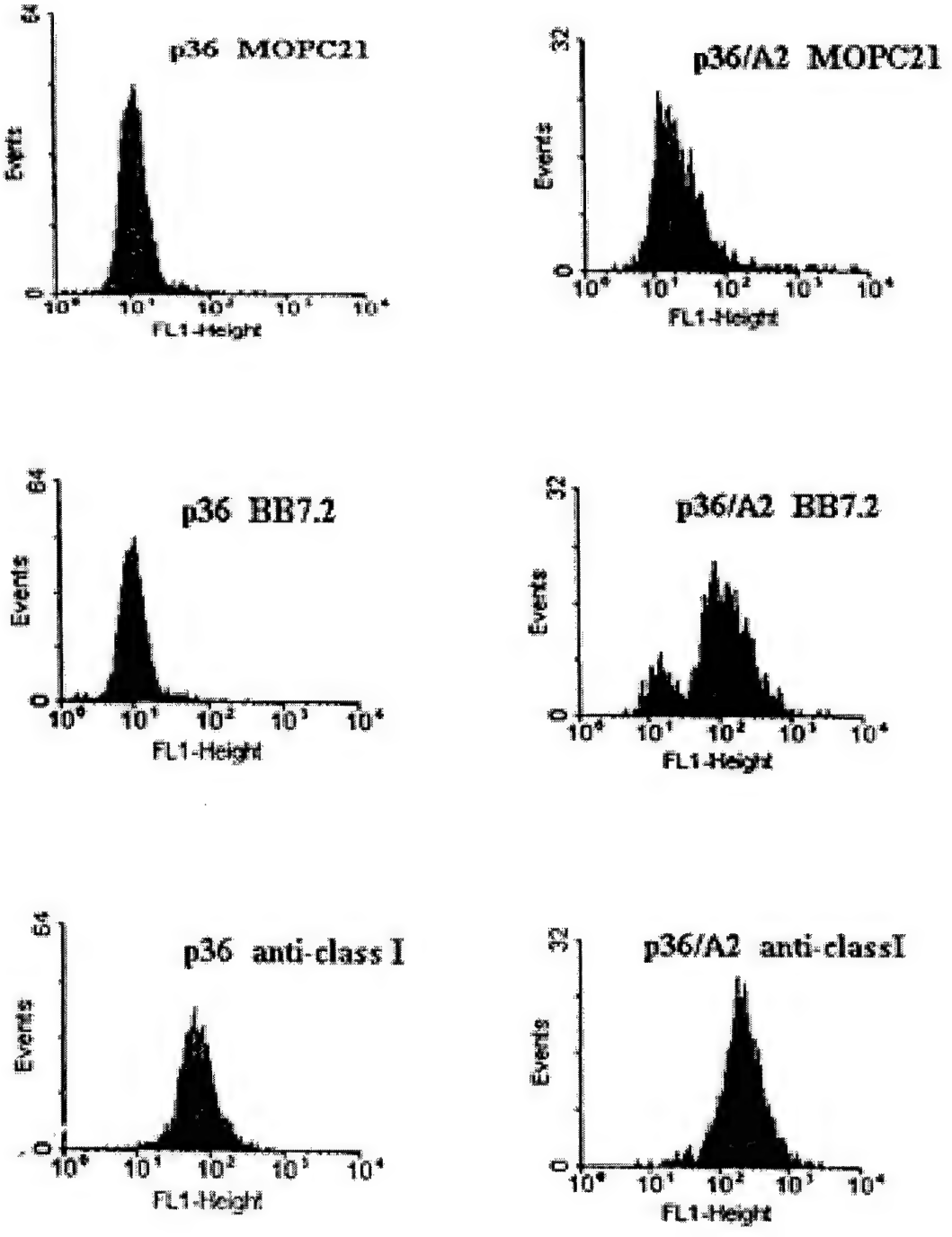


Figure 3. FACScan Analysis of Panned Stably Transfected LNCaP/A2 Subline"46"

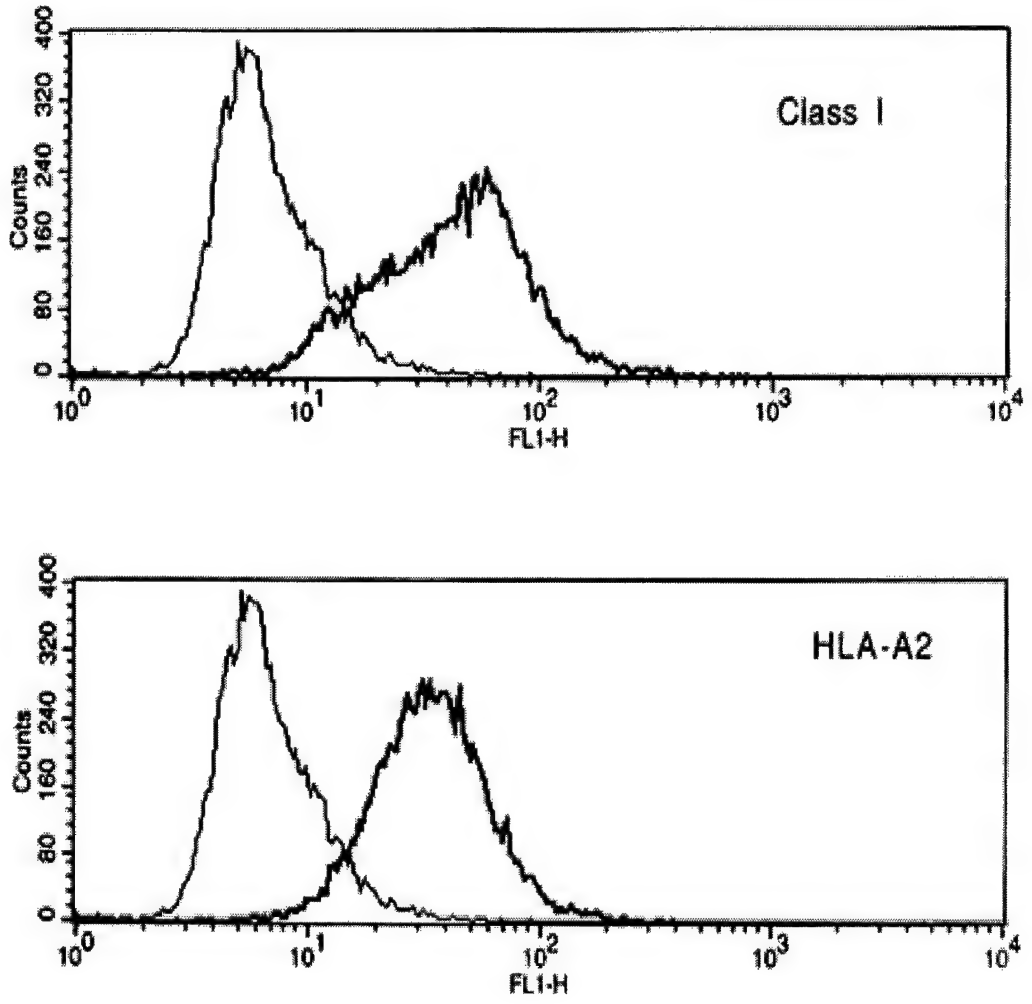


Figure 4. Processing/Cultivation/Characterization of prostate tissue intended for T cell or SEREX antigen identification methods.

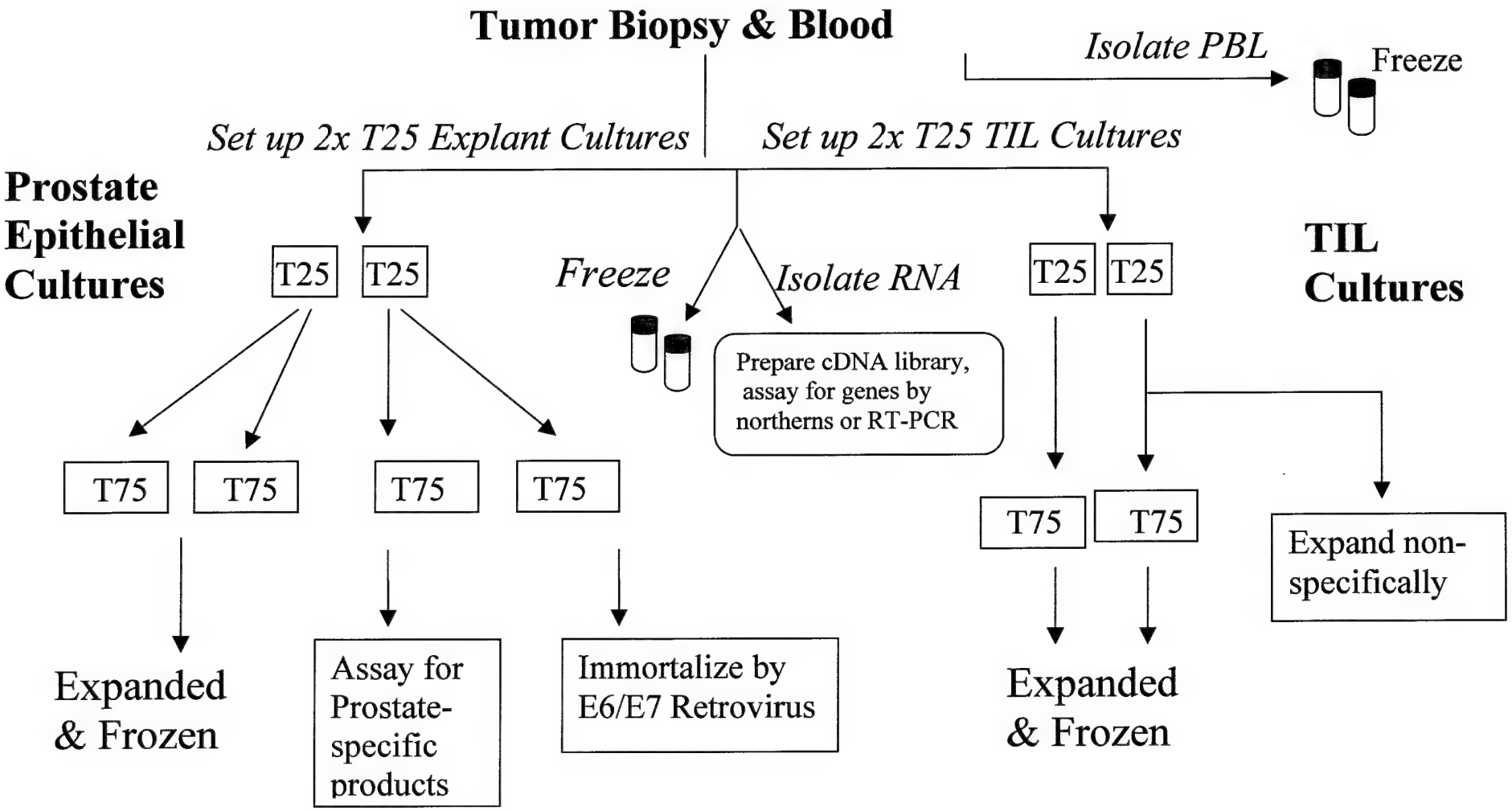


Figure 5. Overview of our approach to SEREX (Serological Identification of Antigens by Recombinant Expression Cloning)

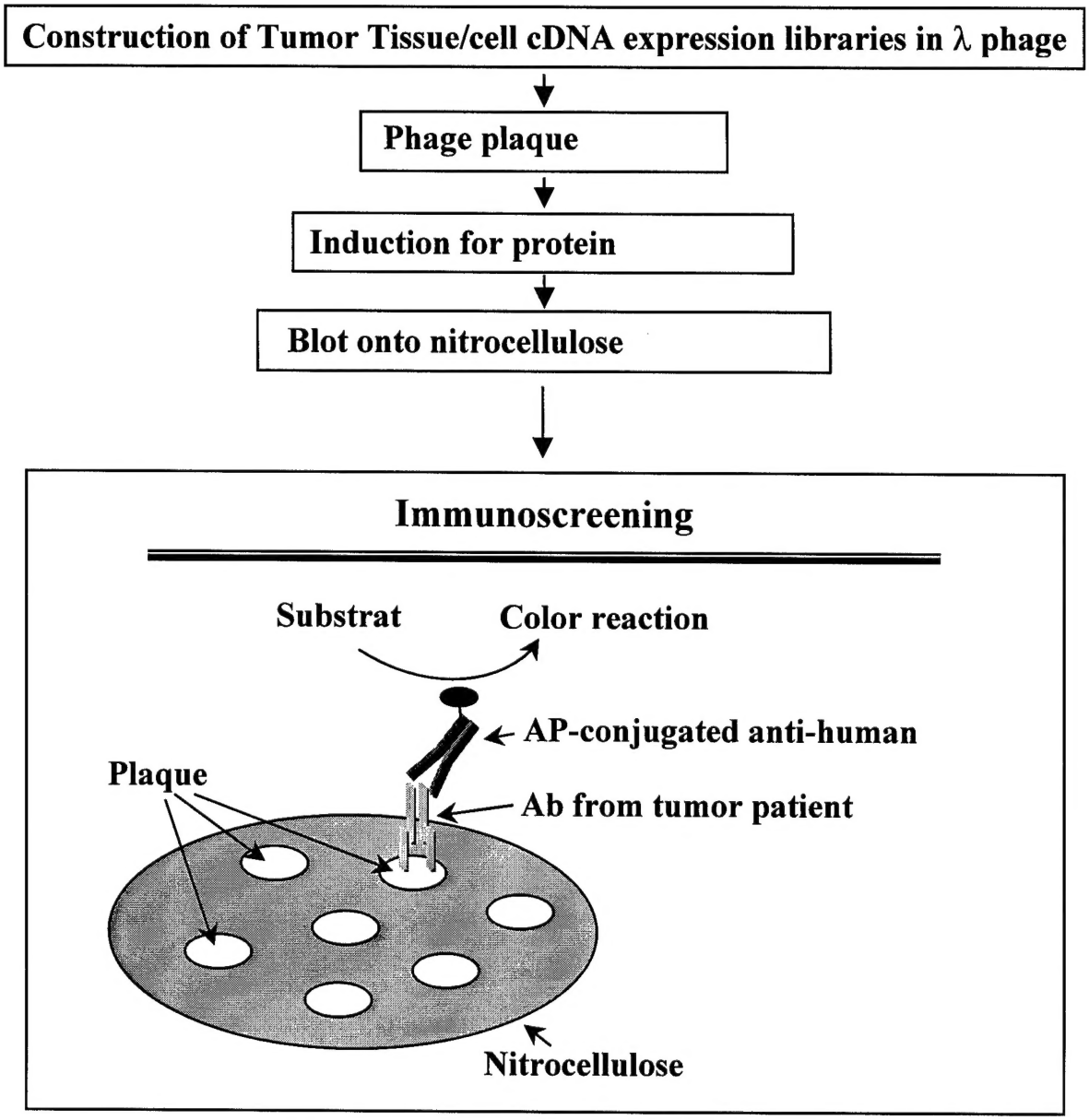


Figure 6. Characterization approach for genes and antigens identified by T cell expression cloning as well as SEREX.

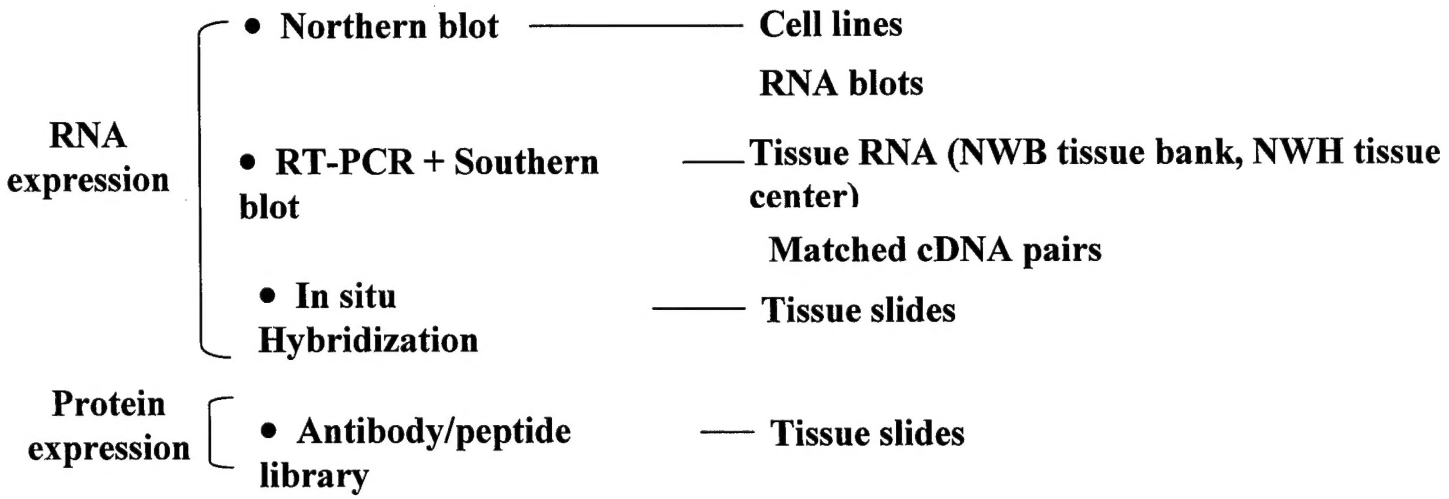


Figure 7. Approach for the expression of proteins in E. coli identified by T cell expression cloning or SEREX

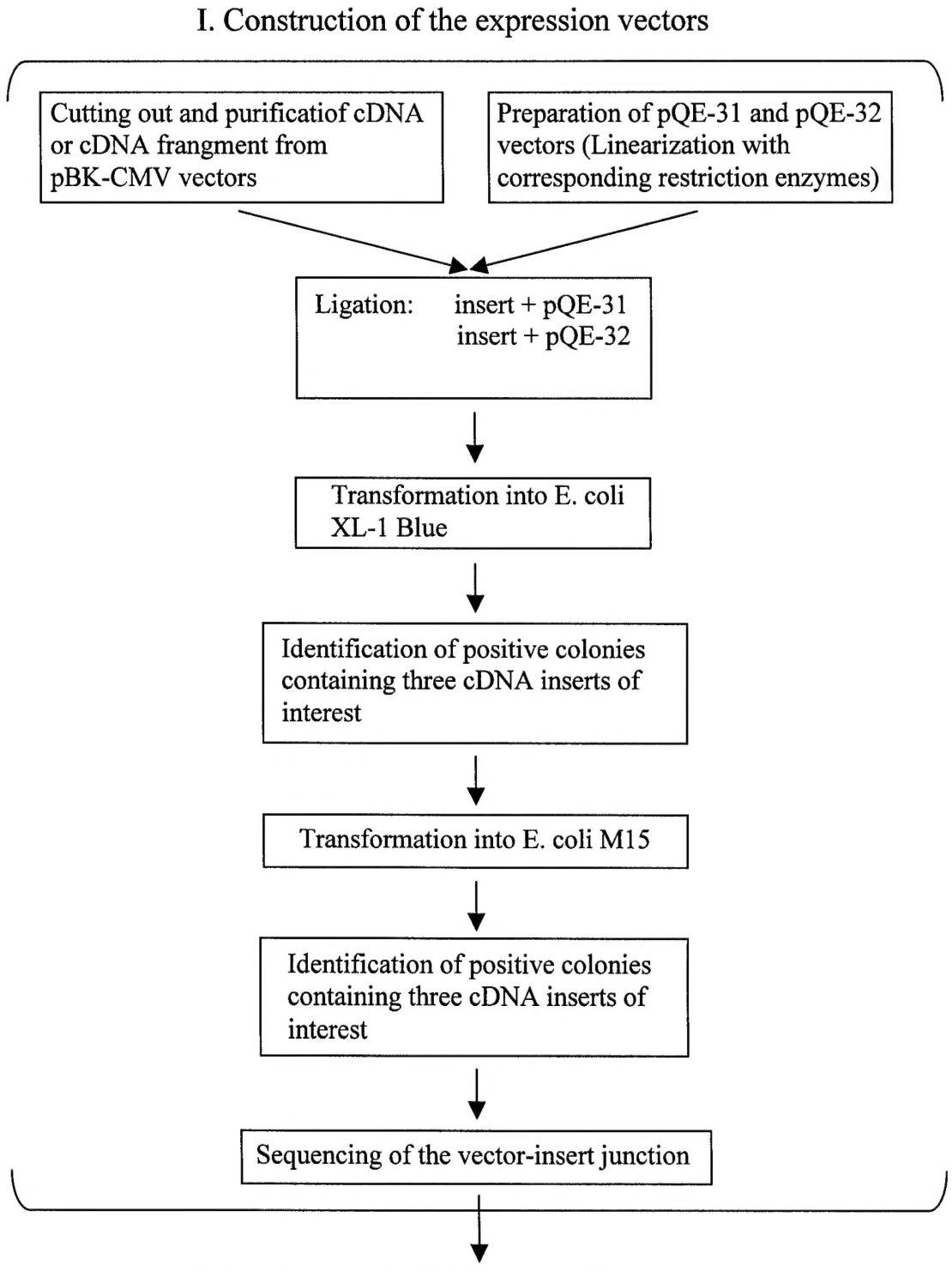
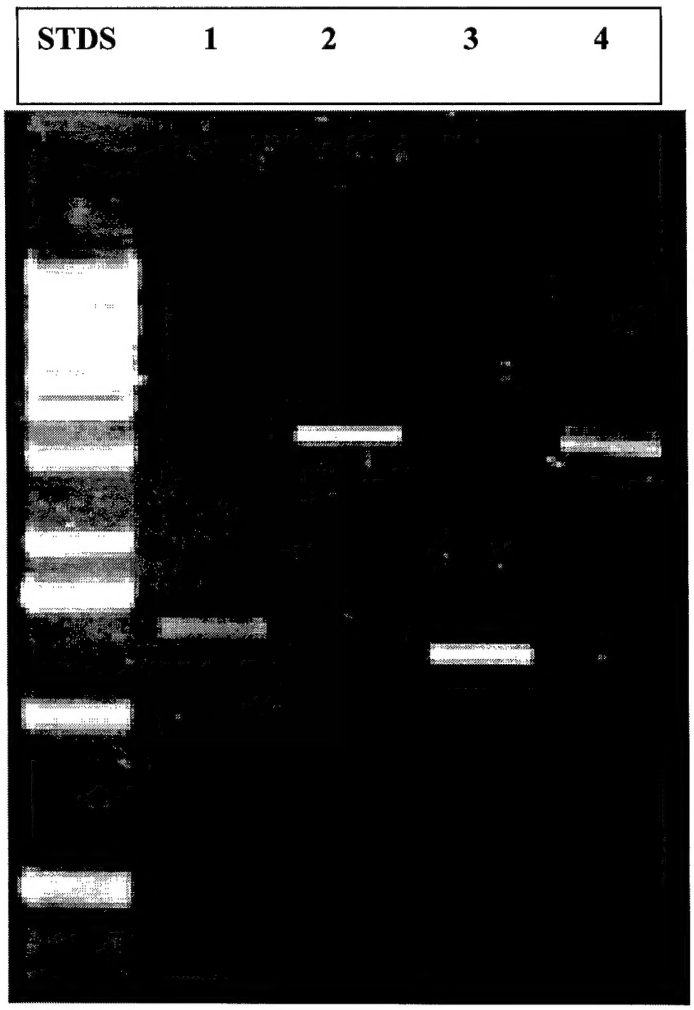


Figure 8. Preparation of P1, P2 inserts and pQE31, 32 vectors for the generation of expression vectors.



Lanes:

1. P1 insert (BamH1/Kpn1)

2. pQE32 vector (BamH1/Kpn1)

3. P2 insert (BamH1/Kpn1)

4. pQE31 vector (BamH1/Kpn1)